

Published in final edited form as:

Mol Microbiol. 2013 September ; 89(5): . doi:10.1111/mmi.12311.

A surface-exposed neuraminidase affects complement resistance and virulence of the oral spirochete *Treponema denticola*

Kurni Kurniyati¹, Weiyan Zhang², Kai Zhang¹, and Chunhao Li^{1,3,*}

¹Department of Oral Biology, the State University of New York at Buffalo, New York 14214

²Department of Pharmaceutical Sciences, the State University of New York at Buffalo, New York 14214

³Department of Microbiology and Immunology, the State University of New York at Buffalo, New York 14214

Abstract

Neuraminidases (sialidases) catalyze the removal of terminal sialic acid from glycoconjugates. Bacterial pathogens often utilize neuraminidases to scavenge host sialic acid, which can be utilized either as a nutrient or as a decorating molecule to disguise themselves from host immune attacks. Herein, a putative neuraminidase (TDE0471) was identified in *Treponema denticola*, an oral spirochete associated with human periodontitis. TDE0471 is a cell surface-exposed exo-neuraminidase that removes sialic acid from human serum proteins; it is required for *T. denticola* to grow in a medium that mimics gingival crevice fluid, suggesting that the spirochete may use sialic acid as a nutrient in vivo. TDE0471 protects *T. denticola* from serum killing by preventing the deposition of membrane attack complexes on the bacterial cell surface. Animal studies revealed that a TDE0471-deficient mutant is less virulent than its parental wild-type strain in BALB/C mice. However, it causes a level of tissue damage similar to the wild type in complement-deficient B6.129S4-*C3^{tm1Crr}/J* mice albeit the damage caused by both bacterial strains is more severe in these transgenic mice. Based on these results, we propose that *T. denticola* has evolved a strategy to scavenge host sialic acid using its neuraminidase, which allows the spirochete to acquire nutrients and evade complement killing.

Keywords

Periodontitis; *Treponema denticola*; Neuraminidase; Sialic acid; Complement system

INTRODUCTION

The complement system plays a central role in host innate immune defense against microbial infections and also functions as a mediator between the innate and adaptive immune responses [for recent reviews, see (Lambris *et al.*, 2008; Ricklin *et al.*, 2010; Zipfel and Skerka, 2009)]. The complement cascade is activated primarily via classical, lectin, and alternative pathways. Activation of the complement system ultimately leads to the formation of the membrane attack complex (MAC), which directly disrupts targeted pathogens via cell lysis. In addition, numerous complement factors that are generated during cascade activation are involved in triggering and orchestrating microbial opsonization, phagocytosis,

*Corresponding author Mailing address: Department of Oral Biology, SUNY at Buffalo, 3435 Main St., Buffalo, NY, 14214-3092, cli9@buffalo.edu; Phone: (716) 829-6014; Fax: (716)-829-3942.

stimulation of the adaptive immune system, and activation of inflammatory cells. Due to its ability to rapidly recognize and eliminate microorganisms, the complement system is considered as a first line of defense against microbial intruders.

Human periodontitis is a prevalent chronic inflammatory disease that damages the supporting connective tissues around teeth and ultimately leads to tooth loss (Darveau, 2010;Pihlstrom *et al.*, 2005). The disease is a polymicrobial infection caused by a diverse group of periodontal pathogens (Hajishengallis *et al.*, 2012a;Pihlstrom *et al.*, 2005). Accumulating evidence suggests that the interactions between the complement system and those periodontal pathogens play a pivotal role in periodontal inflammation and the progression of the disease (Hajishengallis, 2010;Wang *et al.*, 2010;Hajishengallis *et al.*, 2012b). Among those periodontal pathogens, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* (often referred to as 'red-complex' bacteria) have been considered as primary etiological agents of periodontitis (Socransky and Haffajee, 2005;Holt and Ebersole, 2005). This group of bacteria predominantly inhabits periodontal pockets containing gingival crevice fluid (GCF) (Lamont and Jenkinson, 2000;Holt and Ebersole, 2005;Ellen and Galimanas, 2005). The main component (up to 70%) of GCF is plasma, which contains the complement system (Schenkein and Genco, 1977b;Schenkein and Genco, 1977a;Hajishengallis, 2010). Thus, the 'red-complex' bacteria presumably are exposed to continuous immune surveillance mediated by the complement system. However, this group of bacteria has evolved different mechanisms to subvert the killing. *P. gingivalis* suppresses complement killing by producing capsules and degrading key complement factors (i.e., IgG and C3) via cysteine proteases (Popadiak *et al.*, 2007;Schenkein *et al.*, 1995). The oral spirochete *T. denticola* is resistant to the complement killing. Recent studies showed that FhbB, a surface-exposed lipoprotein that binds factor H (FH) and FH-like proteins, contributes to the complement resistance of *T. denticola* (McDowell *et al.*, 2005;McDowell *et al.*, 2009).

Sialic acid is a group of structurally related nine-carbon sugar acids that feature prominently at terminal positions of many eukaryotic surface-exposed glycoconjugates, where they are involved in a wide range of biological processes, including cell-cell interactions and small molecule-cell recognition (Vimr *et al.*, 2004;Severi *et al.*, 2007;Varki, 2007). Several bacterial pathogens have evolved to utilize sialic acid as decorating molecules to modify their surface-exposed macromolecules, such as lipopolysaccharides (LPS) and polysialic acid (PSA) capsules. Such modifications allow bacterial pathogens to disguise themselves and thus circumvent and/or counteract the host's immune responses, including complement killing [for reviews, see (Severi *et al.*, 2007;Vimr *et al.*, 2004;Comstock and Kasper, 2006)]. Neuraminidase (sialidase) is a family of enzymes that catalyzes the removal of terminal sialic acid from glycoconjugates (Achyuthan and Achyuthan, 2001;Powell and Varki, 2001;Roggentin *et al.*, 1993;Vimr, 1994). Various mucosal pathogens, ranging from *Streptococcus pneumoniae* in the airway to *Vibrio cholerae* in the gut, utilize neuraminidases to scavenge host sialic acid (Vimr, 1994;Severi *et al.*, 2007;Galen *et al.*, 1992). In these bacteria, neuraminidases are often associated with their virulence. For instance, the two neuraminidases of *S. pneumoniae* contribute to the progression of infection by promoting pneumococcal brain endothelial cell invasion (Soong *et al.*, 2006;Uchiyama *et al.*, 2009); and the neuraminidase of *Pseudomonas aeruginosa* plays a key role in the initial stages of pulmonary infection (Leprat and Michel-Briand, 1980;Cacalano *et al.*, 1992).

Similar to other mucosal pathogens, the 'red-complex' bacteria also appear to have neuraminidase activity. *P. gingivalis* encodes at least one homologue (PG0352) of neuraminidase. Recent studies showed that PG0352 is involved in biofilm formation, capsule synthesis, serum resistance, and the pathogenicity of *P. gingivalis* (Aruni *et al.*, 2011;Li *et al.*, 2012). *T. forsythia* encodes two neuraminidases (NanH and SiaH). NanH

(TF0035) is a major neuraminidase (Thompson *et al.*, 2009); its deletion mutant fails to attach to and invade human gingival epithelial cells (Honma *et al.*, 2011). *T. denticola* shows neuraminidase activity (Wyss *et al.*, 2004). However, the enzyme(s) conferring this activity and its role in the pathogenicity of *T. denticola* have not been studied. *T. denticola* encodes a putative neuraminidase (TDE0471). In this report, we show experimental evidence that TDE0471 is a neuraminidase that removes sialic acid from human serum glycoconjugates and that it affects nutrient acquisition, complement activation, deposition of MAC, and the virulence of *T. denticola*.

RESULTS

TDE0471 is a neuraminidase

T. denticola appears to have neuraminidase activity (Wyss *et al.*, 2004). However, the enzyme(s) responsible for this activity has not yet been identified. A Blast search of the *T. denticola* ATCC 35405 (hereafter referred to as Td₃₅₄₀₅) genome (Seshadri *et al.*, 2004) using neuraminidase genes from other bacteria suggested that *TDE0471* encodes a putative neuraminidase that consists of 543 amino acids (aa) and has a predicted molecular weight (MW) of 59.8 kDa. The *TDE0471* gene is monocistronic. Transcriptional analyses indicated that it is regulated by a sigma⁷⁰ transcription factor (Fig.S1). The C-terminus (aa 124 to 543) of TDE0471 contains a conserved neuraminidase catalytic domain. Sequence alignment analysis revealed that it contains two conserved functional motifs associated with bacterial neuraminidases (Copley *et al.*, 2001; Crennell *et al.*, 1993): one is the RIP motif and the other is a motif consisting of three Asp-boxes (S-X-D-X-G-X-T-W/F) (Fig.S2), suggesting that TDE0471 is a neuraminidase (hereafter referred to as Td_{neu})

Neuraminidase activity can be detected by a filter paper spot sialidase test (Moncla and Braham, 1989; Moncla *et al.*, 1990). In this assay, neuraminidases cleave 4-MUNANA, a fluorogenic substrate, to produce 4-methylumbelliferone (4-MU), which is fluorescent under ultraviolet light. The substrate treated with recombinant Td_{neu} (rTd_{neu}) produced fluorescence (spot 1, Fig.1A), indicative of neuraminidase activity. The observed activity was completely abolished when the substrate was treated with rTd_{neu}^M (spot 2), a truncated rTd_{neu} in which the C-terminal catalytic domain was deleted. The activity was substantially inhibited when rTd_{neu} was added together with Neu5Ac2en (spots 3 and 4), a neuraminidase-specific inhibitor (Barrett *et al.*, 2011; Trappetti *et al.*, 2009). Further studies demonstrated that rTd_{neu} is active from pH 4.0 to 8.0, with an optimum pH of 5.0 to 6.0 (Fig.1B). It has an affinity toward 4-MUNANA with a K_m of 18.72 ± 4.24 nM and a rapid reaction rate with V_{max} of 0.6 ± 0.03 nmol/nM/min (Fig.1C) under the tested conditions. These results indicate that TDE0471 is a neuraminidase with properties similar to its counterparts in other bacteria.

Td_{neu} is an exo-neuraminidase

Based on substrate specificity, bacterial neuraminidases can be divided into two major classes (Taylor, 1996; Vimr *et al.*, 2004): exo-neuraminidases that cleave 2,3-, 2,6-, and 2,8-glycosidic linkages of terminal sialic acids; and endo-neuraminidases that hydrolyze 2,8-sialosyl linkages in oligo- or poly-sialic acids. The substrate specificity of neuraminidases can be detected by lectin blot assays with human α -1acid glycoprotein (AGP) and three plant lectins: DSA specifically binds to galactose-linked GlcNAc, MAA to 2,3-linked sialic acid, and SNA to 2,6-linked sialic acid (Gut *et al.*, 2008). For this assay, the recombinant NanH protein of *Clostridium perfringens*, a well-studied exo-neuraminidase (Cassidy *et al.*, 1965; Chien *et al.*, 1996), was used as a positive control. As shown in Fig.1D, rTd_{neu} and NanH completely abolished the binding activity of SNA/MAA to AGP (lanes 1 and 2), but had no impact on that of DSA to AGP (top panel). Furthermore, rTd_{neu}^M did not

affect the binding activity of SNA/MAA to AGP (lane 3). These results indicate that Td_{neu} is a NanH-like exo-neuraminidase that is able to cleave both 2,3- and 2,6-linked sialic acid in AGP.

Isolation and characterizations of a *TDE0471* isogenic mutant and its complemented strain

To investigate the function of Td_{neu}, *TDE0471* was inactivated by allelic exchange mutagenesis (Fig.S3A). Erythromycin-resistance colonies that appeared on the plates were first screened by PCR for the presence of the antibiotic resistance gene. One positive clone (Tde471^{mut}) was selected for further characterization by PCR with different pairs of primers. The PCR results showed that the *TDE0471* gene was inactivated as expected (Fig.S4A). Tde471^{mut} was then complemented by inserting the full length *TDE0471* into the erythromycin resistance cassette (*erm^R*) on the chromosome of the mutant, as illustrated in Fig.S3B. The resultant complemented colonies became erythromycin sensitive and gentamicin resistant. Tde471^{com}, a complemented clone, was selected for further characterization. Immunoblotting analysis using a specific antibody against Td_{neu} (Td_{neu}) showed that Td_{neu} was absent in Tde471^{mut} and restored to the wild-type level in Tde471^{com} (Fig.2A). The paper spot test using whole cell lysates of *T. denticola* strains showed that neuraminidase activity was detected in Td₃₅₄₀₅ and Tde471^{com}, but not in Tde471^{mut} (Fig. 2B). These results indicated that the *TDE0471* gene product and enzymatic activity were abrogated in Tde471^{mut} and restored in Tde471^{com}. In addition, as no residual neuraminidase activity was detected in Tde471^{mut} (lane 2, Fig.2B), it is most likely that Td_{neu} is the only neuraminidase of *T. denticola*.

Td_{neu} is a cell surface-exposed protein

Signal peptide prediction using the SignalP 4.0 server suggested that the N-terminal 24 aa of Td_{neu} is a signal peptide (Fig.S5), implying that it is likely secreted. To test this prediction, the wild-type cells were first treated with proteinase K (PK) and the resultant cell pellets were analyzed by immunoblots. After the treatment, no trace of Td_{neu} was detected in the cell pellets (Fig.3A), suggesting that Td_{neu} resides on the cell surface and can be readily degraded by PK. This proposition was further confirmed by IFA studies using Td_{neu}, in which strong fluorescence signals were observed in the wild type cells, but not in the mutant cells (Fig.3B).

T. denticola surface-exposed proteins (e.g., the major surface protein, Msp) are often dissociated from cell surfaces and released to the culture media (Fenno *et al.*, 1998; Bian *et al.*, 2005). To determine if this is the case for Td_{neu}, supernatants prepared from the *T. denticola* cultures were analyzed by immunoblots to detect Td_{neu} and by the filter paper spot assay to test for the enzymatic activity. Consistent with the hypothesis, the protein and enzymatic activity were detected in the supernatants of Td₃₅₄₀₅ and Tde471^{com}, but not in that of the mutant (Fig.3C, D). To rule out the possibility that the detected protein and enzymatic activity are due to cell lysis during the growth and sample preparation, FlaA, a flagellar sheath protein in the periplasmic space of *T. denticola* (Ruby *et al.*, 1997), was used as a sample control; no trace of FlaA was detected in the supernatants (Fig.3C). Collectively, these results indicate that Td_{neu} is a surface-exposed protein that is readily dissociated from the cells and released into its growth milieu.

Td_{neu} cleaves sialic acid from human serum proteins

Total sialic acid in the plasma is abundant (approximately 2 mM). Under normal physiological conditions, almost all sialic acids (99.9%) are bound to a diverse range of glycoproteins and glycolipids (often named sialoglycoconjugates) (Ponnio *et al.*, 1999; Sillanauke *et al.*, 1999). To determine if Td_{neu} is able to desialylate those sialoglycoconjugates, human serum was incubated with rTd_{neu} and then probed with SNA

and MAA. SNA and MAA bound to a variety of serum sialoglycoconjugates (Fig.4). The observed binding activities showed a dose-dependent reduction when the serum was treated with different amounts of rTd_{neu} for 1 hour (Fig.4A), and the binding activities were almost completely abolished when the serum was co-incubated with 0.2 µg rTd_{neu} for 3 hours (Fig. 4B, C). Of note, there was no desialylation activity observed in the samples treated with rTd_{neu}^M (Fig.4B, C). In addition, the treatment of the serum with rTd_{neu} had no impact on the binding activity of Concanavalin A (ConA) (Fig.4D), suggesting that rTd_{neu} has no proteolytic activity against the serum proteins. These results indicate that the corresponding 2,3- and 2,6-linked terminal sialic acid residues on the serum sialoglycoconjugates were cleaved by Td_{neu}. This activity is consistent with the catalytic feature of Td_{neu} as an exo-neuraminidase, as shown in Fig.2.

The Tde471^{mut} fails to grow in the serum growth medium

Several bacteria are able to utilize sialic acid as a nutrient when other carbohydrates are limited (Vimr *et al.*, 2004;Severi *et al.*, 2007). In the TYGVS medium, Td₃₅₄₀₅, Tde471^{mut} and Tde471^{com} strains exhibited similar growth patterns (Fig.5A), suggesting that the lack of Td_{neu} has no significant impact on *T. denticola* growth in a nutrient-enriched medium. As mentioned earlier, *T. denticola* primarily lives in the GCF, which contains 50~70% serum (Schenkein and Genco, 1977b;Schenkein and Genco, 1977a). To mimic this growth condition, the *T. denticola* strains were cultivated in serum growth medium that contains 50% heat-inactivated rabbit serum. In this medium, both Td₃₅₄₀₅ and Tde471^{com} were able to grow and reached approximately 10⁸ cells/ml at the stationary phase. However, Tde471^{mut} completely failed to grow (Fig.5B). Interestingly, the mutant recovered its growth ability and reached the same cell density as Td₃₅₄₀₅ and Tde471^{com} at the stationary phase when the medium was supplemented with 100 or 500 µM of exogenous N-acetylneuraminic acid (Neu5Ac, a well-studied sialic acid and the most abundant one; Fig.5B) (Vimr *et al.*, 2004; Comb and Roseman, 1960). These results indicate that *T. denticola* may require Td_{neu} to release sialic acid from the serum, which can be further metabolized to provide essential nutrients for its growth in vivo.

Td_{neu} affects serum bactericidal activity against *T. denticola*

Several complement factors (e.g., C1q, IgG, and FH) are glycosylated and contain sialic acid. In some cases, sialylation is essential for their biological activities, such as the complement activation and formation of MAC (Ritchie *et al.*, 2002;Varki, 2007). To determine if Td_{neu} affects serum bactericidal activity against *T. denticola*, serum killing assays were conducted. Similar to a recent report (McDowell *et al.*, 2011), Td₃₅₄₀₅ was resistant to the serum killing and its average survival rate was 87% following 1-hour incubation in 25% normal human serum (NHS). Compared to Td₃₅₄₀₅, Tde471^{mut} was more vulnerable to the killing – its survival rate decreased to 39% (Fig.6). The diminished resistance was fully restored when the mutant was complemented (Fig.6). These results indicate that Td_{neu} acts to attenuate the serum bactericidal activity against *T. denticola*.

Tde471^{mut} exhibits enhanced complement deposition on the cell surface

The hallmark of complement-dependent killing is the formation of MAC (C5b-9) on bacterial cell surfaces (Lambris *et al.*, 2008;Ricklin *et al.*, 2010;Vimr and Lichtensteiger, 2002). Tde471^{mut} exhibited decreased serum resistance (Fig.6). Conceivably, the loss of Td_{neu} may enhance complement deposition and formation of MAC on the mutant cells, which would make Tde471^{mut} more vulnerable to the serum killing. To test this hypothesis, complement deposition on the Td₃₅₄₀₅, Tde471^{mut}, and Tde471^{com} strains was measured and compared. As shown in Fig.7A, SDS-PAGE analyses showed that various proteins were evident in the serum-incubated mutant cells, but not in the samples of Td₃₅₄₀₅ and Tde471^{com} after 20 min incubation in NHS. Notably, these proteins disappeared when the

mutant cells were treated with EDTA (Fig.7A), a chelating agent that blocks the complement cascade activation (Berger *et al.*, 1985). In addition, the immunoblotting analyses using a rat serum against *T. denticola* (Fig.7B) and a polyclonal antibody against Msp (data not shown) demonstrated the presence of similar amounts of *T. denticola* proteins in the experimental samples (Fig.7B). Collectively, these results suggest that the proteins present on the mutant were derived from the complement system, not from the bacterial cells.

This proposition was further confirmed by immunoblotting analyses with specific antibodies against C3, C9, and C5b-9 complex (Fig.7C-E). For C3 antibody (C3), four products were detected in the serum-incubated Tde471^{mut} cells, but not in the serum-incubated Td₃₅₄₀₅ and Tde471^{com} cells. The sizes of the detected bands are similar to those of C3-related products (Fig.7C), including C3b chain (119 kDa) and chain (75 kDa), and factor I-mediated C3b cleaved products (iC3b-1, 68 kDa; 2, 43 kDa) (Barnes and Weiss, 2001; Ben and Klimpel, 2008). A similar pattern was observed when the samples were probed with antibodies against C9 (C9) and C5b-9 (C5b-9) – in the serum-incubated mutant cells, mono-C9 (71 kDa) and poly-C9 (>1,000 kDa) were detected by C9 (Fig.7D); and two glycosylated C9 products were detected by C5b-9 (Fig.7E). In contrast, none of these proteins was evident in the serum-incubated Td₃₅₄₀₅ and Tde471^{com} cells. Taken together, these results indicate that Td_{neu} impairs the complement activation and deposition. This constitutes a novel protection mechanism of *T. denticola* from the serum killing.

Td_{neu} contributes to the virulence of *T. denticola*

The importance of Td_{neu} in the virulence of *T. denticola* was further assessed in vivo using a previously reported mouse skin infection model (Kesavalu *et al.*, 1997). For this study, each BALB/C mouse was subcutaneously injected with 10⁹ *T. denticola* cells at its dorsal site. Skin abscesses caused by the spirochetes were measured at ten days post-injection. Compared to the Td₃₅₄₀₅ and Tde471^{com} strains, the tissue damage caused by Tde471^{mut} was less ($p < 0.01$), which was reflected by smaller skin abscesses on mice injected with the mutant (Fig.8A). The average size of the abscesses induced by Td₃₅₄₀₅ was $71.6 \pm 6.9 \text{ mm}^2$ (n=5). The average size was reduced to $37.5 \pm 2.0 \text{ mm}^2$ (n=5) in the mutant and was restored to the wild-type level in Tde471^{com} ($67.5 \pm 4.7 \text{ mm}^2$, n=4).

To determine if the complement system has any impact on the pathogenicity of *T. denticola*, a similar experiment was repeated in B6.129S4-*C3^{tm1Crr}/J*, a complement-deficient mouse strain (Wessels *et al.*, 1995). The tissue damage caused by both Td₃₅₄₀₅ and Tde471^{mut} was more severe in B6.129S4-*C3^{tm1Crr}/J* mice than in its parental wild-type strain C57BL/6 (Fig. 8B). In the mutant mice, the average sizes of skin abscesses induced by Td₃₅₄₀₅ and Tde471^{mut} were $118.8 \pm 15.3 \text{ mm}^2$ (n=5) and $88.6 \pm 8.4 \text{ mm}^2$ (n=5), respectively, which are significantly ($p < 0.01$) larger than what is observed in C57BL/6 mice ($55.9 \pm 5.0 \text{ mm}^2$, n=5). The average size of the abscesses induced by Tde471^{mut} is smaller than that induced by Td₃₅₄₀₅ (Fig.8B), but it is not statistically significant ($p > 0.1$). These results along with the in vitro studies presented above indicate that the immune defense mediated by the complement system affects the pathogenicity of *T. denticola* and that Td_{neu} is an important virulence factor for complement evasion in vivo.

DISCUSSION

Do the neuraminidases of 'red-complex' bacteria share similar biochemical characteristics?

As mentioned earlier, the 'red-complex' bacteria primarily inhabit the gingival crevices bathed in plasma, which contains a high concentration of sialic acid that is bound to a

diverse range of glycans. In this unique niche, it would be a survival advantage if a microorganism can utilize host sialic acid. In this regard, it is not surprising that all three 'red-complex' bacteria possess neuraminidases. In addition, several lines of evidence have suggested that those neuraminidases (PG0352, TF0035, and Td_{neu}) have similar biochemical features. First, they are all dual-domain proteins (Li *et al.*, 2012;Thompson *et al.*, 2009). The neuraminidase domains are located at their C-terminal regions while the N-termini have different domains. PG0352 has a peptidoglycan-binding domain (aa 30 to 180). The N-terminus of Td_{neu} contains a Listeria-Bacteroides-Repeat domain that is often found in internalin families (CDD212296) of *Listeria* species and is implicated in cell adhesion and invasion (Dramsi *et al.*, 1993). Second, they are active over a wide pH range (e.g., TF0035 is active from pH 4.5 to 8.0, with an optimum pH of 5.5) (Thompson *et al.*, 2009). Td_{neu} is active in a similar pH range to TF0035 (Fig.1B). Third, they all show exo-neuraminidase activity. Similar to PG0352 and TF0035 (Li *et al.*, 2012;Thompson *et al.*, 2009), Td_{neu} is able to remove 2,3- and 2,6-linked sialic acid of AGP (Fig.1D). Finally, they all contain a signal peptide, suggesting they are most likely secreted and/or surface-localized. Previous studies have shown that TF0035 is both surface-localized and secreted (Honma *et al.*, 2011). Similar to TF0035, Td_{neu} is also surface-exposed and secreted (Fig.3). These biochemical characteristics are compatible with the gingival crevice microenvironment – the pH of GCF in healthy individuals is about 6.9 (Eggert *et al.*, 1991;Bickel and Cimasoni, 1985). In addition, 2,3- and 2,6-linked sialic acids are the most abundant and are ubiquitous at the termini of plasma sialoglycoconjugates (Ponnio *et al.*, 1999;Crocker *et al.*, 2007;Varki, 2008). These features may allow these enzymes to maintain their optimal activities in terms of withstanding pH fluctuation and acquiring sufficient substrates in the periodontal pockets.

Does *T. denticola* utilize sialic acid as a nutrient?

Several bacteria use sialic acid as an alternative nutrient when other carbon resources are limited (Vimr *et al.*, 2004;Severi *et al.*, 2007). The plasma in the gingival crevices contains a variety of sialoglycoconjugates that can be desialylated by Td_{neu} (Fig.4). The released free sialic acid presumably can be transported and metabolized within *T. denticola* cells. This can explain why Td₃₅₄₀₅ is able to grow in the serum medium but Tde471^{mut} cannot (Fig.5). One intriguing question remaining to be answered is how *T. denticola* metabolizes sialic acid and converts it to other nutrients. In *Escherichia coli*, NanA first metabolizes Neu5Ac and then releases ManNAc and pyruvate. ManNAc is ultimately converted to fructose-6-phosphate and ammonia via a series of reactions catalyzed by NanK, NanE, NagB and NagA [for review, see (Vimr *et al.*, 2004)]. However, the genes encoding these enzymes are absent in the genome of *T. denticola* (Seshadri *et al.*, 2004), indicating that a similar catabolic pathway does not exist in the spirochete. The metabolism of *T. denticola* is poorly understood and the catabolism of sialic acid in anaerobes has not yet been documented. It is possible that *T. denticola* and other anaerobes may have evolved a novel catabolic pathway to metabolize sialic acid. Recent studies have suggested that *T. forsythia* might use Neu5Ac and *N*-acetyl-glucosamine (NAG) to produce *N*-acetylmuramic acid (NAM) (Roy *et al.*, 2011), a key component of cell wall peptidoglycan. A similar scenario may also occur in *T. denticola*.

How does *T. denticola* escape complement killing?

In this report, we found that Tde471^{mut} was more vulnerable to the serum killing (Fig.6). However, under the tested condition, approximately 39% of the mutant cells were still viable, indicating that other mechanisms are also involved in the complement resistance of *T. denticola*. Dentilisin, a surface protease of *T. denticola*, hydrolyzes C3b (Yamazaki *et al.*, 2006) and cleaves FH (McDowell *et al.*, 2011). Since C3b plays a key role in the complement cascade activation (Lambris *et al.*, 2008;Ricklin *et al.*, 2010), it has been previously postulated that dentilisin may contribute to the serum resistance of *T. denticola*

(Yamazaki *et al.*, 2006). However, McDowell et al recently reported that dentilisin has no impact on the serum resistance of *T. denticola* (McDowell *et al.*, 2011). This observation is contradictory to its activity of cleaving C3b and FH; and more comprehensive studies are needed to further elucidate its role in the serum resistance of *T. denticola*. In addition, recent reports described that FhbB, an FH-binding protein, is involved in complement evasion (McDowell *et al.*, 2011). Conceivably, these factors may constitute a complex and redundant network that protects *T. denticola* from the complement killing, ensuring that the spirochete can survive and thrive in the oral flora while facing the constant immune surveillance that is mediated by the complement system.

How does Td_{neu} protect *T. denticola* from the serum killing?

Previous reports have shown that FhbB, an FH-binding protein, protects *T. denticola* from serum killing (McDowell *et al.*, 2005; McDowell *et al.*, 2011). In *Neisseria gonorrhoeae*, sialylation increases the binding activity of FH to LPS, which results in higher serum resistance (Ram *et al.*, 1998b; Ram *et al.*, 1998a). We sought to determine if a similar scenario accounts for the serum resistance of *T. denticola* (e.g., the free sialic acid released by Td_{neu} is utilized to modify FhbB, which results in increased binding of FH to FhbB). To test this hypothesis, an affinity-ligand-binding immunoblotting (ALBI) assay was carried out (Fig.S6). The results showed that FH bound to both Td₃₅₄₀₅ and Tde471^{mut} in a similar pattern, but not to FhbB^{mut}, a previously constructed *fhbB* isogenic mutant (McDowell *et al.*, 2011). This indicates that the loss of Td_{neu} has no impact on FH binding to Tde471^{mut}. Thus, it is unlikely that Td_{neu} affects the serum resistance of *T. denticola* by acting on FhbB.

Several pathogenic bacteria (e.g., *Campylobacter jejuni*, *Haemophilus influenza*, and *Neisseria* spp) acquire serum resistance by the incorporation of sialic acid into their surface macromolecules (i.e., LPS and capsule) (Figueira *et al.*, 2007; Ram *et al.*, 1998b; Madico *et al.*, 2007; Avril *et al.*, 2006; Vimr and Lichtensteiger, 2002; Severi *et al.*, 2007). However, *T. denticola* lacks both LPS and capsule (Seshadri *et al.*, 2004). If a similar mechanism exists in *T. denticola*, the organism must utilize other surface molecules rather than capsule and LPS. Proteomic analysis disclosed that at least four proteins had either changed expression levels or had altered molecular weights in Tde471^{mut} (Li, et al, unpublished data). These proteins were Msp (TDE0405), oligopeptide-binding protein (TDE0985), OppA (TDE1071), and a putative outer-membrane protein (TDE2602). Msp and OppA are two virulence factors of *T. denticola* (Fenno *et al.*, 1998; Fenno *et al.*, 2000). These proteins could be potential candidates for modification by sialic acid. Consistent with this possibility, a recent proteomic analysis showed that Msp and OppA have different forms (Veith *et al.*, 2009), suggesting that they are probably modified (e.g., glycosylation). Furthermore, *T. denticola* has a novel type of outer membrane lipid that contains lipoteichoic acids (Schultz *et al.*, 1998), and genomic information suggests that it may produce surface polysaccharides (Seshadri *et al.*, 2004). These two molecules are potential candidates for sialylation. We are currently employing different approaches to investigate if these candidate molecules are sialylated or not.

Summary

The evidence described in this report along with previous studies has demonstrated that 'red-complex' bacteria have evolved to employ neuraminidases to scavenge host sialic acid. This can benefit the pathogens at least in two ways: nutrient acquisition and immune evasion. Accumulating evidence has shown that neuraminidases play critical roles in the physiology and pathogenicity of 'red-complex' bacteria (Roy *et al.*, 2011; Aruni *et al.*, 2011; Li *et al.*, 2012). These neuraminidases are surfaced-exposed and share similar biochemical features. Given these facts, the neuraminidases are ideal targets for developing new therapeutic agents for intervention and prevention of periodontitis (e.g., designing specific inhibitors against

neuraminidases). In addition, the surfaces of host immune system cells contain a family of sialic-acid-binding immunoglobulin-like lectins (Siglecs), which play critical roles in regulating the functions of the cells in innate and adaptive immune systems via glycan recognition (Varki, 2007; Crocker *et al.*, 2007; Varki, 2008). It is intriguing to consider the potential effects that neuraminidases could impose on innate and adaptive immunity regulated by the Siglecs. Furthermore, sialylation is essential for the anti-inflammatory activity of human plasma immunoglobulin G (also referred to as intravenous IgG, IVIG), which is often utilized to treat a variety of human hematological and immunological disorders (Nimmerjahn and Ravetch, 2008). Recent studies have shown that desialylation of IVIG not only depletes its anti-inflammatory activity but also potentially leads to generation of pathogenic antibodies (Kaneko *et al.*, 2006). Our preliminary studies suggested that Td_{neu} is able to cleave sialic acid from IVIG (data not shown). It is even more intriguing to consider the potential impact of neuraminidases on IgG in the GCF and the role of IgG in the pathogenesis of periodontitis as well as systemic diseases that are associated with periodontitis.

EXPERIMENTAL PROCEDURES

Ethics statement

All animal experimentation was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Human serum samples were collected from healthy volunteers. The protocols for animal studies and the use of human serum were approved by the Institutional Animal Care and Use Committee (permit number: ORB 23068Y) and the Human Subjects Review Committee (permit number: ORB0321006E) of the State University of New York at Buffalo, respectively.

Bacterial strains and culture conditions

T. denticola ATCC 35405 (wild type) (Seshadri *et al.*, 2004) was used in this report. Cells were grown either in tryptone-yeast extract-gelatin-volatile fatty acids-serum medium (TYGVS) (Ohta *et al.*, 1986) or in 50% heat-inactivated (56°C for 30 min) rabbit serum diluted in PBS (phosphate buffered saline, pH 7.4) supplemented with 6 µg/ml thiamine pyrophosphate (TPP) (designated as serum growth medium) at 37°C with an atmosphere of 85% nitrogen, 5% carbon dioxide, and 10% hydrogen. *T. denticola* isogenic mutants were grown with appropriate antibiotic(s) for selective pressure as needed: erythromycin (70 µg/ml) and/or gentamicin (20 µg/ml). *E. coli* TOP10 strain (Invitrogen, Carlsbad, CA) was used for DNA cloning; BL21 Star™ (DE3) (Invitrogen) and M15 (Qiagen, Valencia, CA) strains were used for preparing recombinant proteins. The *E. coli* strains were cultured in lysogeny broth (LB) supplemented with appropriate concentrations of antibiotics.

Preparation of recombinant TDE0471 and its antiserum

To prepare a recombinant protein for enzymatic analysis, the full-length *TDE0471* gene was PCR amplified with primers P₁/P₂ using *Vent* DNA polymerase (New England Biolabs, Ipswich, MA). The obtained PCR product was cloned into pET101/D-TOPO vector (Invitrogen), which encodes a six-histidine tag at the C-terminus. The resulting plasmid was then transformed into BL21 Star (DE3) cells. The expression of *TDE0471* was induced using 1 mM isopropyl -D-1-thiogalactosidase (IPTG). The recombinant protein was purified using Ni-NTA agarose (Qiagen) under native conditions as previously described (Sze *et al.*, 2011; Li *et al.*, 2012). The purified protein was then dialyzed in a buffer containing 20 mM Tris-HCl at 4°C overnight using 6.0 kDa molecular weight cut-off Spectra/Por® dialysis bags (Spectrum Laboratories, Rancho Dominguez, CA). The concentration of purified protein was determined using a Bio-Rad Protein Assay Kit (Bio-

Rad Laboratories, Hercules, CA). A similar method was used to prepare a truncated TDE0471 recombinant protein without the C-terminal catalytic domain (from aa 324 to 543). This protein was used either as an antigen for production of TDE0471 antibody or as a control in the biochemical analyses. For antibody production, 5 mg purified truncated TDE0471 protein was used to immunize rats (2.5 mg for each animal) following a standard immunization procedure as previously described (Sze and Li, 2011). The primers for preparation of the recombinant proteins are listed in Table S1.

Electrophoresis and immunoblotting analyses

Sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and immunoblotting using the enhanced chemiluminescent (ECL) detection system were carried out as described before (Sze and Li, 2011). *T. denticola* cells were harvested at the late-logarithmic-phase ($\sim 10^8$ cells/ml). Equal amounts of whole cell lysates (10-50 μ g) were separated on SDS-PAGE gels and then transferred to PVDF membranes (Bio-Rad). The immunoblots were probed with specific antibodies against various proteins and developed using horseradish peroxidase-coupled secondary antibody with an ECL luminol assay. The antibodies against the *T. denticola* flagellin protein (FlaA) and Msp were gifts from J. C. Fenno (University of Michigan). The polyclonal antibodies against *T. denticola* were raised in this study by immunizing rats twice with the wild-type whole cells. A monoclonal antibody against human FH was purchased from Thermo Scientific (Thermo Scientific, Rockford, IL). A polyclonal against C3 and monoclonal antibodies against C9 and C5b-9 complex were purchased from Abcam (Abcam Inc., Cambridge, MA).

Enzyme assays

A previously documented filter paper spot sialidase assay was used to detect the neuraminidase activity (Moncla and Braham, 1989; Li *et al.*, 2012). Briefly, the recombinant TDE0471, the whole cell lysates, or the supernatants from the *T. denticola* cultures were incubated with 2'-4-methylumbelliferyl- β -D-N-acetyl-neuraminic acid (4-MUNANA) (Sigma-Aldrich), a fluorogenic neuraminidase substrate. Fluorescence was measured using a ChemiDoc XRS imaging system (Bio-Rad) with an excitation wavelength of 302 nm and an emission wavelength of 548 nm. To test the neuraminidase specificity, 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid (Neu5Ac2en), a neuraminidase inhibitor (Barrett *et al.*, 2011), was included in the assay. To determine the optimal pH for the enzymatic activity, the filter paper spot assay was carried out at various pH values (ranging from 3.0 to 9.0). The neuraminidase kinetics analysis was carried out as previously described (Thompson *et al.*, 2009; Gut *et al.*, 2008). Briefly, 3.342 nM purified recombinant TDE0471 protein was incubated with various amounts of 4-MUNANA (ranging from 0.02 to 0.25 mM) in a reaction buffer containing 70 mM sodium citrate (pH 7.0) at 37°C for 15 minutes. The saturation curves were fitted to Michaelis-Menten kinetics using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA), and the K_m and V_{max} were calculated.

Lectin blot analysis

A previously described lectin blot analysis (Gut *et al.*, 2008) was used to determine the substrate specificity of TDE0471. Briefly, 50 μ l aliquots containing 1 μ g human α -1 acid glycoprotein (AGP), 0.7 nM recombinant TDE0471 (0.7 nM) or 0.0064 U *Clostridium perfringens* neuraminidase NanH (Sigma-Aldrich), and 10 mM potassium phosphate (pH 7.0) were incubated overnight at 37°C. After the incubation, the samples were separated on SDS-PAGE gels, and then transferred to PVDF membranes (Bio-Rad). The resultant blots were probed with biotin-labeled lectins (Vector Laboratories, Inc., Burlingame, CA) from three plants: *Datura stramonium* (DSA), *Sambucus nigra* (SNA), and *Maackia amurensis* (MAA) (Crowley *et al.*, 1984). These three lectins specifically recognize different carbohydrates – SNA binds to α -2,6-linked sialic acid, MAA to α -2,3-linked sialic acid, and

DSA to galactose-linked GlcNAc. The final blots were incubated with streptavidin-horseradish peroxidase conjugate and developed with the ECL luminol assay (Thermo Scientific).

Treatment of human serum with recombinant TDE0471

Human serum samples were collected from healthy laboratory donors. Freshly drawn blood was allowed to clot and separated by centrifugation at $2,000 \times g$ for 20 min. The serum samples were aliquoted in small volumes and stored at -80°C . Each aliquot was thawed only once. To determine if TDE0471 cleaves sialic acid from sialoglycoconjugates present in the serum, 0.15% (final concentration in the reactions) or 1.5% normal human serum (NHS) was incubated with various amounts of recombinant TDE0471 in a reaction buffer (10 mM sodium phosphate, pH 5.0) for 1 to 3 hrs at 37°C . After incubation, the treated samples were then subjected to SDS-PAGE and immunoblotting analyses. The blots were first blocked in $1 \times$ Carbo-Free blocking solution (Vector Laboratories) containing 0.05% Tween-20 and then incubated with biotinylated lectins in an incubation buffer ($0.2 \times$ Carbo-Free solution, 0.05% Tween-20) for 1 h at room temperature. The lectins used here include SNA, MAA (Vector Laboratories), and Concanavalin A (ConA, Sigma-Aldrich). The resulting blots were washed four times with PBS-T buffer (PBS, 0.05% Tween-20) and then incubated with streptavidin-horseradish peroxidase conjugate. After incubation, the blots were washed four times with PBS-T buffer and developed with the ECL luminol assay.

Constructing a TDE0471 isogenic mutant and its cognate complemented strain

The construct *TDE0471::erm^R* (Fig.S3A) was used to inactivate *TDE0471* via allelic exchange recombination. The construct *Erm^R::TDE0471* (Fig.S3B) was used for *cis*-complementation of *TDE0471* mutant. To construct *TDE0471::erm^R*, a previously described erythromycin resistant cassette (*erm^R*) (Li *et al.*, 1996) and a DNA fragment containing the entire *TDE0471* gene were amplified by PCR using primers P₅/P₆ and P₇/P₈, respectively. The resulting amplicons were cloned into pGEM-T vector. The cloned *erm^R* cassette was then released by EcoRI from the plasmid and inserted into *TDE0471* at the same cleavage site, yielding *TDE0471::erm^R*. *Erm^R::TDE0471* was constructed by two step PCR and DNA cloning. The 5'-portion of *erm^R* and a previously constructed gentamicin resistance cassette (*aacC1*) (Bian *et al.*, 2012) were PCR amplified with primers P₉/P₁₀ and P₁₁/P₁₂, respectively, and then fused together with primers P₉/P₁₂, generating Fragment 1 (Fig.S3B). The 3'-portion of *erm^R* and a DNA fragment containing the full length of *TDE0471* and its upstream promoter sequence were PCR amplified with primers P₁₅/P₁₆ and P₁₃/P₁₄, respectively, and then fused together by PCR using primers P₁₃/P₁₆, generating Fragment 2 (Fig.S3). The primers used are listed in Table S1. The two obtained DNA fragments were cloned into the pGEM-T easy vector and then fused together at the engineered XbaI cleavage site. Preparation of *T. denticola* competent cells, electroporation, and cell plating were carried out as previously described (Li *et al.*, 1996; Bian *et al.*, 2011). The resultant antibiotic-resistant clones were first characterized by PCR and the selected positive clones were further confirmed by immunoblotting analysis.

Preparations of proteinase K-treated T. denticola cells and culture supernatants

The proteinase K treatment was performed as previously described (Brooks *et al.*, 2006). Briefly, 10 ml of stationary-phase *T. denticola* cultures (5×10^8 cells/ml) were harvested by centrifugation at $2,000 \times g$ for 15 min. The harvested cells were washed three times in PBS. They were then resuspended in 2.5 ml PBS containing 600 μg of proteinase K (Sigma-Aldrich) and incubated at 37°C for 1 h. The reactions were stopped by adding 10 μl of 10 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), followed by centrifugation at $2,000 \times g$ for 15 min. The obtained cell pellets were washed twice in PBS and subjected to SDS-PAGE and immunoblotting analyses. *T. denticola* culture supernatants were prepared as

previously described (Rosen *et al.*, 1995). Briefly, 400 ml of the late-logarithmic-phase *T. denticola* cultures (10^8 cells/ml) were centrifuged and the obtained culture supernatants were filtered through 0.2 μm -pore-size filters to further remove the remaining cells. The filtered supernatants were then dialyzed in 4 liters of 50 mM Tris-HCl buffer (pH 7.8) at 4°C overnight using 3.5 kDa molecular weight cut-off Spectra/Por® dialysis membranes and further concentrated to 4 ml.

Immunofluorescence assay (IFA)

IFA was conducted to determine the surface location of TDE0471 as previously described with slight modifications (Xu *et al.*, 2011). Briefly, 1.5 ml of *T. denticola* cultures were harvested, washed one time with PBS buffer, and then treated with methanol at -20°C for 1 hour. The resultant cells were then placed on poly-L-lysine-coated cover slips, and allowed to fully air dry. The obtained cover slips were first incubated in a blocking solution (2% BSA in PBS, pH 7.5) for 1 hour, followed by incubation in the blocking solution containing 1:100 diluted Td_{neu} for 1 hour at room temperature. Finally, the cover slips were washed five times with PBS, incubated with the secondary goat anti-mouse Texas red antibody (Invitrogen) for 1 hour at room temperature, washed with PBS, and mounted in 40% glycerol for image processing as described previously (Xu *et al.*, 2011).

Measuring *T. denticola* growth rates

To measure the growth rates, 5 μl of the late-logarithmic-phase *T. denticola* cultures (10^8 cells/ml) were inoculated into 5 ml of the TYGVS medium, the serum growth medium, or the serum medium supplemented with either 100 or 500 $\mu\text{g}/\text{ml}$ of *N*-acetyl-neuraminic acid (Neu5Ac). *T. denticola* cells in the cultures were enumerated every 24 h or 48 h using a Petroff Hausser counting chamber (Hausser Scientific, Horsham, PA). Each growth curve is representative of at least three independent cultures, and the results are represented as the mean of cell numbers \pm standard error of the mean (SEM).

Serum killing assay

The serum killing assay was conducted as previously described (McDowell *et al.*, 2011; Li *et al.*, 2012). Briefly, 75 μl of *T. denticola* cultures (containing 10^6 spirochete cells) were first mixed with 25 μl of either normal human serum (NHS) or heat-inactivated serum (HIS) and then incubated at 37°C in the anaerobic chamber for 1 h. After incubation, the number of living spirochete cells was enumerated as described above. The living cells were determined by whether they were motile during the observation time (at least 1 min). The average survival rates (the number of living cells in NHS / the number of living cells in HIS) were calculated from at least three independent experiments, and the results are represented as the mean of survival rates \pm standard error of the mean (SEM).

Complement deposition assay

The complement deposition assay was carried out as previously described (Barnes and Weiss, 2001). For this assay, 75 μl of *T. denticola* cultures (containing 10^6 cells from the late-logarithmic-phase cultures) were mixed with 25 μl of NHS and then incubated for 20 min at 37°C in the anaerobic chamber. As controls, 10 mM ethylenediamine-tetraacetic acid (EDTA) and 10 mM MgCl₂-ethylene glycol-bis (2-aminoethyl ether)-*N,N,N,N*-tetraacetic acid (Mg-EGTA) were included in the assay either to block activation of the complement pathways or to selectively activate the alternative pathway. After the incubation, the reactions were incubated on ice for 1 min and then ice-cold PBS containing 10 mM EDTA was added to stop complement activation. The cells were collected by centrifugation. The cell pellets were washed three times with ice-cold PBS and resuspended in 50 μl PBS. After adding 20 μl Laemmli sample buffer, the resultant samples were boiled for 5 min, adjusted

to 100 μ l, and finally 5 μ l of samples were subjected to SDS-PAGE and immunoblotting analyses.

Affinity-ligand-binding immunoblotting (ALBI) assay

The ALBI assay was carried out as previously described (McDowell *et al.*, 2005). Briefly, the late-logarithmic-phase *T. denticola* cultures were harvested, separated on 15% SDS-PAGE gels, and transferred to PVDF membranes. The blots were incubated with purified human factor H (FH) (Complement Technology Inc., Tyler, TX). The bound FH was detected by a monoclonal antibody against FH, following the standard immunoblotting procedure described above.

Animal studies

A previously documented mouse skin abscess model was used to assess the virulence of *T. denticola* (Kesavalu *et al.*, 1997). For the animal studies, ~6-8 week-old BALB/C mice, complement-deficient mice (B6.129S4-*C3^{tm1Crr}/J*) (Wessels *et al.*, 1995) and its parental wild-type stain C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were included. Each mouse (at least 4 mice per bacterial strain) received a single subcutaneous injection on its posterior dorsolateral surface with 200 μ l PBS containing 1×10^9 bacterial cells. After infection, mice were monitored for symptoms of infection on a daily basis for a total of ten days. The diameters of the observed abscesses were measured with a caliper gauge. Each abscess was measured at least three times from different angles and average sizes were calculated (area = r^2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank C. J. Fenno for providing antibodies against FlaA and Msp, R. Marconi for providing the FhbB mutant strain, and S. Ruhl for providing Concanavalin A. This research was supported by Public Health Service Grants DE019667 and DE023080 to C. Li.

Reference List

- Achyuthan KE, Achyuthan AM. Comparative enzymology, biochemistry and pathophysiology of human exo-alpha-sialidases (neuraminidases). *Comp Biochem Physiol B Biochem Mol Biol.* 2001; 129:29–64. [PubMed: 11337249]
- Aruni W, Vanterpool E, Osbourne D, Roy F, Muthiah A, Dou Y, Fletcher HM. Sialidase and sialoglycoproteases can modulate virulence in *Porphyromonas gingivalis*. *Infect Immun.* 2011; 79:2779–2791. [PubMed: 21502589]
- Avril T, Wagner ER, Willison HJ, Crocker PR. Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on *Campylobacter jejuni* lipooligosaccharides. *Infect Immun.* 2006; 74:4133–4141. [PubMed: 16790787]
- Barnes MG, Weiss AA. BrkA protein of *Bordetella pertussis* inhibits the classical pathway of complement after C1 deposition. *Infect Immun.* 2001; 69:3067–3072. [PubMed: 11292725]
- Barrett S, Mohr PG, Schmidt PM, McKimm-Breschkin JL. Real time enzyme inhibition assays provide insights into differences in binding of neuraminidase inhibitors to wild type and mutant influenza viruses. *PLoS One.* 2011; 6:e23627. [PubMed: 21858186]
- Ben NA, Klimpel GR. Subversion of complement activation at the bacterial surface promotes serum resistance and opsonophagocytosis of *Francisella tularensis*. *J Leukoc Biol.* 2008; 84:77–85. [PubMed: 18430786]

- Berger M, Birx DL, Wetzler EM, O'Shea JJ, Brown EJ, Cross AS. Calcium requirements for increased complement receptor expression during neutrophil activation. *J Immunol.* 1985; 135:1342–1348. [PubMed: 3159792]
- Bian J, Fenno JC, Li C. Development of a modified gentamicin resistance cassette for genetic manipulation of the oral spirochete *Treponema denticola*. *Appl Environ Microbiol.* 2012; 78:2059–2062. [PubMed: 22247130]
- Bian J, Shen H, Tu Y, Yu A, Li C. The riboswitch regulates a thiamine pyrophosphate ABC transporter of the oral spirochete *Treponema denticola*. *J Bacteriol.* 2011; 193:3912–3922. [PubMed: 21622748]
- Bian XL, Wang HT, Ning Y, Lee SY, Fenno JC. Mutagenesis of a novel gene in the *prcA*-*prtP* protease locus affects expression of *Treponema denticola* membrane complexes. *Infect Immun.* 2005; 73:1252–1255. [PubMed: 15664975]
- Bickel M, Cimasoni G. The pH of human crevicular fluid measured by a new microanalytical technique. *J Periodontal Res.* 1985; 20:35–40. [PubMed: 3156233]
- Brooks CS, Vuppala SR, Jett AM, Akins DR. Identification of *Borrelia burgdorferi* outer surface proteins. *Infect Immun.* 2006; 74:296–304. [PubMed: 16368984]
- Cacalano G, Kays M, Saiman L, Prince A. Production of the *Pseudomonas aeruginosa* neuraminidase is increased under hyperosmolar conditions and is regulated by genes involved in alginate expression. *J Clin Invest.* 1992; 89:1866–1874. [PubMed: 1601994]
- Cassidy JT, Jourdain GW, Roseman S. The sialic acids. VI Purification and properties of sialidase from *Clostridium perfringens*. *J Biol Chem.* 1965; 240:3501–3506. [PubMed: 4284295]
- Chien CH, Shann YJ, Sheu SY. Site-directed mutations of the catalytic and conserved amino acids of the neuraminidase gene, *nanH*, of *Clostridium perfringens* ATCC 10543. *Enzyme Microb Technol.* 1996; 19:267–276. [PubMed: 8987487]
- Comb DG, Roseman S. The sialic acids. I. The structure and enzymatic synthesis of N-acetylneuraminic acid. *J Biol Chem.* 1960; 235:2529–2537. [PubMed: 13811398]
- Comstock LE, Kasper DL. Bacterial glycans: key mediators of diverse host immune responses. *Cell.* 2006; 126:847–850. [PubMed: 16959564]
- Copley RR, Russell RB, Ponting CP. Sialidase-like Asp-boxes: sequence-similar structures within different protein folds. *Protein Sci.* 2001; 10:285–292. [PubMed: 11266614]
- Crennell SJ, Garman EF, Laver WG, Vimr ER, Taylor GL. Crystal structure of a bacterial sialidase (from *Salmonella typhimurium* LT2) shows the same fold as an influenza virus neuraminidase. *Proc Natl Acad Sci U S A.* 1993; 90:9852–9856. [PubMed: 8234325]
- Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. *Nat Rev Immunol.* 2007; 7:255–266. [PubMed: 17380156]
- Crowley JF, Goldstein IJ, Arnarp J, Lonngren J. Carbohydrate binding studies on the lectin from *Datura stramonium* seeds. *Arch Biochem Biophys.* 1984; 231:524–533. [PubMed: 6203486]
- Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol.* 2010; 8:481–490. [PubMed: 20514045]
- Dramsi S, Kocks C, Forestier C, Cossart P. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator *prfA*. *Mol Microbiol.* 1993; 9:931–941. [PubMed: 7934921]
- Eggert FM, Drewell L, Bigelow JA, Speck JE, Goldner M. The pH of gingival crevices and periodontal pockets in children, teenagers and adults. *Arch Oral Biol.* 1991; 36:233–238. [PubMed: 1908671]
- Ellen RP, Galimanas VB. Spirochetes at the forefront of periodontal infections. *Periodontol.* 2000; 2005; 38:13–32. [PubMed: 15853935]
- Fenno JC, Hannam PM, Leung WK, Tamura M, Uitto VJ, McBride BC. Cytopathic effects of the major surface protein and the chymotrypsinlike protease of *Treponema denticola*. *Infect Immun.* 1998; 66:1869–1877. [PubMed: 9573063]
- Fenno JC, Tamura M, Hannam PM, Wong GW, Chan RA, McBride BC. Identification of a *Treponema denticola* OppA homologue that binds host proteins present in the subgingival environment. *Infect Immun.* 2000; 68:1884–1892. [PubMed: 10722578]

- Figueira MA, Ram S, Goldstein R, Hood DW, Moxon ER, Pelton SI. Role of complement in defense of the middle ear revealed by restoring the virulence of nontypeable *Haemophilus influenzae* *siaB* mutants. *Infect Immun*. 2007; 75:325–333. [PubMed: 17088344]
- Galen JE, Ketley JM, Fasano A, Richardson SH, Wasserman SS, Kaper JB. Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. *Infect Immun*. 1992; 60:406–415. [PubMed: 1730470]
- Gut H, King SJ, Walsh MA. Structural and functional studies of *Streptococcus pneumoniae* neuraminidase B: An intramolecular trans-sialidase. *FEBS Lett*. 2008; 582:3348–3352. [PubMed: 18775704]
- Hajishengallis G. Complement and periodontitis. *Biochem Pharmacol*. 2010; 80:1992–2001. [PubMed: 20599785]
- Hajishengallis G, Darveau RP, Curtis MA. The keystone-pathogen hypothesis. *Nat Rev Microbiol*. 2012a; 10:717–725. [PubMed: 22941505]
- Hajishengallis G, Krauss JL, Liang S, McIntosh ML, Lambris JD. Pathogenic microbes and community service through manipulation of innate immunity. *Adv Exp Med Biol*. 2012b; 946:69–85. [PubMed: 21948363]
- Holt SC, Ebersole JL. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the “red complex”, a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol* 2000. 2005; 38:72–122. [PubMed: 15853938]
- Honma K, Mishima E, Sharma A. Role of *Tannerella forsythia* NanH sialidase in epithelial cell attachment. *Infect Immun*. 2011; 79:393–401. [PubMed: 21078857]
- Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science*. 2006; 313:670–673. [PubMed: 16888140]
- Kesavalu L, Walker SG, Holt SC, Crawley RR, Ebersole JL. Virulence characteristics of oral treponemes in a murine model. *Infect Immun*. 1997; 65:5096–5102. [PubMed: 9393801]
- Lambris JD, Ricklin D, Geisbrecht BV. Complement evasion by human pathogens. *Nat Rev Microbiol*. 2008; 6:132–142. [PubMed: 18197169]
- Lamont RJ, Jenkinson HF. Subgingival colonization by *Porphyromonas gingivalis*. *Oral Microbiol Immunol*. 2000; 15:341–349. [PubMed: 11154429]
- Leprat R, Michel-Briand Y. Extracellular neuraminidase production by a strain of *Pseudomonas aeruginosa* isolated from cystic fibrosis. *Ann Microbiol (Paris)*. 1980; 131B:209–222. [PubMed: 6781392]
- Li C, Kurniyati, Hu B, Bian J, Sun J, Zhang W, et al. Abrogation of neuraminidase reduces biofilm formation, capsule biosynthesis, and virulence of *Porphyromonas gingivalis*. *Infect Immun*. 2012; 80:3–13. [PubMed: 22025518]
- Li H, Ruby J, Charon N, Kuramitsu H. Gene inactivation in the oral spirochete *Treponema denticola*: construction of an *flgE* mutant. *J Bacteriol*. 1996; 178:3664–3667. [PubMed: 8655571]
- Madico G, Ngampasutadol J, Gulati S, Vogel U, Rice PA, Ram S. Factor H binding and function in sialylated pathogenic neisseriae is influenced by gonococcal, but not meningococcal, porin. *J Immunol*. 2007; 178:4489–4497. [PubMed: 17372007]
- McDowell JV, Frederick J, Miller DP, Goetting-Minesky MP, Goodman H, Fenno JC, Marconi RT. Identification of the primary mechanism of complement evasion by the periodontal pathogen, *Treponema denticola*. *Mol Oral Microbiol*. 2011; 26:140–149. [PubMed: 21375704]
- McDowell JV, Huang B, Fenno JC, Marconi RT. Analysis of a unique interaction between the complement regulatory protein factor H and the periodontal pathogen *Treponema denticola*. *Infect Immun*. 2009; 77:1417–1425. [PubMed: 19204088]
- McDowell JV, Lankford J, Stamm L, Sadlon T, Gordon DL, Marconi RT. Demonstration of factor H-like protein 1 binding to *Treponema denticola*, a pathogen associated with periodontal disease in humans. *Infect Immun*. 2005; 73:7126–7132. [PubMed: 16239506]
- Moncla BJ, Braham P. Detection of sialidase (neuraminidase) activity in *Actinomyces* species by using 2'-(4-methylumbelliferyl)alpha-D-N-acetylneuraminic acid in a filter paper spot test. *J Clin Microbiol*. 1989; 27:182–184. [PubMed: 2643620]
- Moncla BJ, Braham P, Hillier SL. Sialidase (neuraminidase) activity among gram-negative anaerobic and capnophilic bacteria. *J Clin Microbiol*. 1990; 28:422–425. [PubMed: 2108991]

- Nimmerjahn F, Ravetch JV. Anti-inflammatory actions of intravenous immunoglobulin. *Annu Rev Immunol.* 2008; 26:513–533. [PubMed: 18370923]
- Ohta K, Makinen KK, Loesche WJ. Purification and characterization of an enzyme produced by *Treponema denticola* capable of hydrolyzing synthetic trypsin substrates. *Infect Immun.* 1986; 53:213–220. [PubMed: 3013780]
- Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet.* 2005; 366:1809–1820. [PubMed: 16298220]
- Ponnio M, Alho H, Nikkari ST, Olsson U, Rydberg U, Sillanaukee P. Serum sialic acid in a random sample of the general population. *Clin Chem.* 1999; 45:1842–1849. [PubMed: 10508132]
- Popadiak K, Potempa J, Riesbeck K, Blom AM. Biphasic effect of gingipains from *Porphyromonas gingivalis* on the human complement system. *J Immunol.* 2007; 178:7242–7250. [PubMed: 17513773]
- Powell LD, Varki AP. Sialidases. *Curr Protoc Mol Biol.* 2001; Chapter 17(Unit17)
- Ram S, McQuillen DP, Gulati S, Elkins C, Pangburn MK, Rice PA. Binding of complement factor H to loop 5 of porin protein IA: a molecular mechanism of serum resistance of nonsialylated *Neisseria gonorrhoeae*. *J Exp Med.* 1998a; 188:671–680. [PubMed: 9705949]
- Ram S, Sharma AK, Simpson SD, Gulati S, McQuillen DP, Pangburn MK, Rice PA. A novel sialic acid binding site on factor H mediates serum resistance of sialylated *Neisseria gonorrhoeae*. *J Exp Med.* 1998b; 187:743–752. [PubMed: 9480984]
- Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol.* 2010; 11:785–797. [PubMed: 20720586]
- Ritchie GE, Moffatt BE, Sim RB, Morgan BP, Dwek RA, Rudd PM. Glycosylation and the complement system. *Chem Rev.* 2002; 102:305–319. [PubMed: 11841245]
- Roggentin P, Schauer R, Hoyer LL, Vimr ER. The sialidase superfamily and its spread by horizontal gene transfer. *Mol Microbiol.* 1993; 9:915–921. [PubMed: 7934919]
- Rosen G, Naor R, Rahamim E, Yishai R, Sela MN. Proteases of *Treponema denticola* outer sheath and extracellular vesicles. *Infect Immun.* 1995; 63:3973–3979. [PubMed: 7558307]
- Roy S, Honma K, Douglas CW, Sharma A, Stafford GP. Role of sialidase in glycoprotein utilization by *Tannerella forsythia*. *Microbiology.* 2011; 157:3195–3202. [PubMed: 21885482]
- Ruby JD, Li H, Kuramitsu H, Norris SJ, Goldstein SF, Buttle KF, Charon NW. Relationship of *Treponema denticola* periplasmic flagella to irregular cell morphology. *J Bacteriol.* 1997; 179:1628–1635. [PubMed: 9045823]
- Schenkein HA, Fletcher HM, Bodnar M, Macrina FL. Increased opsonization of a prtH-defective mutant of *Porphyromonas gingivalis* W83 is caused by reduced degradation of complement-derived opsonins. *J Immunol.* 1995; 154:5331–5337. [PubMed: 7730636]
- Schenkein HA, Genco RJ. Gingival fluid and serum in periodontal diseases. I. Quantitative study of immunoglobulins, complement components, and other plasma proteins. *J Periodontol.* 1977a; 48:772–777. [PubMed: 73582]
- Schenkein HA, Genco RJ. Gingival fluid and serum in periodontal diseases. II. Evidence for cleavage of complement components C3, C3 proactivator (factor B) and C4 in gingival fluid. *J Periodontol.* 1977b; 48:778–784. [PubMed: 338883]
- Schultz CP, Wolf V, Lange R, Mertens E, Wecke J, Naumann D, Zahringer U. Evidence for a new type of outer membrane lipid in oral spirochete *Treponema denticola*. Functioning permeation barrier without lipopolysaccharides. *J Biol Chem.* 1998; 273:15661–15666. [PubMed: 9624160]
- Seshadri R, Myers GS, Tettelin H, Eisen JA, Heidelberg JF, Dodson RJ, et al. Comparison of the genome of the oral pathogen *Treponema denticola* with other spirochete genomes. *Proc Natl Acad Sci U S A.* 2004; 101:5646–5651. [PubMed: 15064399]
- Severi E, Hood DW, Thomas GH. Sialic acid utilization by bacterial pathogens. *Microbiology.* 2007; 153:2817–2822. [PubMed: 17768226]
- Sillanaukee P, Ponnio M, Jaaskelainen IP. Occurrence of sialic acids in healthy humans and different disorders. *Eur J Clin Invest.* 1999; 29:413–425. [PubMed: 10354198]
- Socransky SS, Haffajee AD. Periodontal microbial ecology. *Periodontol 2000.* 2005; 38:135–187. [PubMed: 15853940]

- Soong G, Muir A, Gomez MI, Waks J, Reddy B, Planet P, et al. Bacterial neuraminidase facilitates mucosal infection by participating in biofilm production. *J Clin Invest*. 2006; 116:2297–2305. [PubMed: 16862214]
- Sze CW, Li C. Inactivation of *bb0184*, which encodes carbon storage regulator A, represses the infectivity of *Borrelia burgdorferi*. *Infect Immun*. 2011; 79:1270–1279. [PubMed: 21173314]
- Sze CW, Morado DR, Liu J, Charon NW, Xu H, Li C. Carbon storage regulator A (CsrA(Bb)) is a repressor of *Borrelia burgdorferi* flagellin protein FlaB. *Mol Microbiol*. 2011; 82:851–864. [PubMed: 21999436]
- Taylor G. Sialidases: structures, biological significance and therapeutic potential. *Curr Opin Struct Biol*. 1996; 6:830–837. [PubMed: 8994884]
- Thompson H, Homer KA, Rao S, Booth V, Hosie AH. An orthologue of *Bacteroides fragilis* NanH is the principal sialidase in *Tannerella forsythia*. *J Bacteriol*. 2009; 191:3623–3628. [PubMed: 19304852]
- Trappetti C, Kadioglu A, Carter M, Hayre J, Iannelli F, Pozzi G, et al. Sialic acid: a preventable signal for pneumococcal biofilm formation, colonization, and invasion of the host. *J Infect Dis*. 2009; 199:1497–1505. [PubMed: 19392624]
- Uchiyama S, Carlin AF, Khosravi A, Weiman S, Banerjee A, Quach D, et al. The surface-anchored NanA protein promotes pneumococcal brain endothelial cell invasion. *J Exp Med*. 2009; 206:1845–1852. [PubMed: 19687228]
- Varki A. Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. *Nature*. 2007; 446:1023–1029. [PubMed: 17460663]
- Varki A. Sialic acids in human health and disease. *Trends Mol Med*. 2008; 14:351–360. [PubMed: 18606570]
- Veith PD, Dashper SG, O'Brien-Simpson NM, Paolini RA, Orth R, Walsh KA, Reynolds EC. Major proteins and antigens of *Treponema denticola*. *Biochim Biophys Acta*. 2009; 1794:1421–1432. [PubMed: 19501677]
- Vimr E, Lichtensteiger C. To sialylate, or not to sialylate: that is the question. *Trends Microbiol*. 2002; 10:254–257. [PubMed: 12088651]
- Vimr ER. Microbial sialidases: does bigger always mean better? *Trends Microbiol*. 1994; 2:271–277. [PubMed: 7981969]
- Vimr ER, Kalivoda KA, Deszo EL, Steenbergen SM. Diversity of microbial sialic acid metabolism. *Microbiol Mol Biol Rev*. 2004; 68:132–153. [PubMed: 15007099]
- Wang M, Krauss JL, Domon H, Hosur KB, Liang S, Magotti P, et al. Microbial hijacking of complement-toll-like receptor crosstalk. *Sci Signal*. 2010; 3:ra11. [PubMed: 20159852]
- Wessels MR, Butko P, Ma M, Warren HB, Lage AL, Carroll MC. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. *Proc Natl Acad Sci U S A*. 1995; 92:11490–11494. [PubMed: 8524789]
- Wyss C, Moter A, Choi BK, Dewhirst FE, Xue Y, Schupbach P, et al. *Treponema putidum* sp. nov., a medium-sized proteolytic spirochaete isolated from lesions of human periodontitis and acute necrotizing ulcerative gingivitis. *Int J Syst Evol Microbiol*. 2004; 54:1117–1122. [PubMed: 15280279]
- Xu H, Raddi G, Liu J, Charon NW, Li C. Chemoreceptors and flagellar motors are subterminally located in close proximity at the two cell poles in spirochetes. *J Bacteriol*. 2011; 193:2652–2656. [PubMed: 21441520]
- Yamazaki T, Miyamoto M, Yamada S, Okuda K, Ishihara K. Surface protease of *Treponema denticola* hydrolyzes C3 and influences function of polymorphonuclear leukocytes. *Microbes Infect*. 2006; 8:1758–1763. [PubMed: 16815066]
- Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. *Nat Rev Immunol*. 2009; 9:729–740. [PubMed: 19730437]

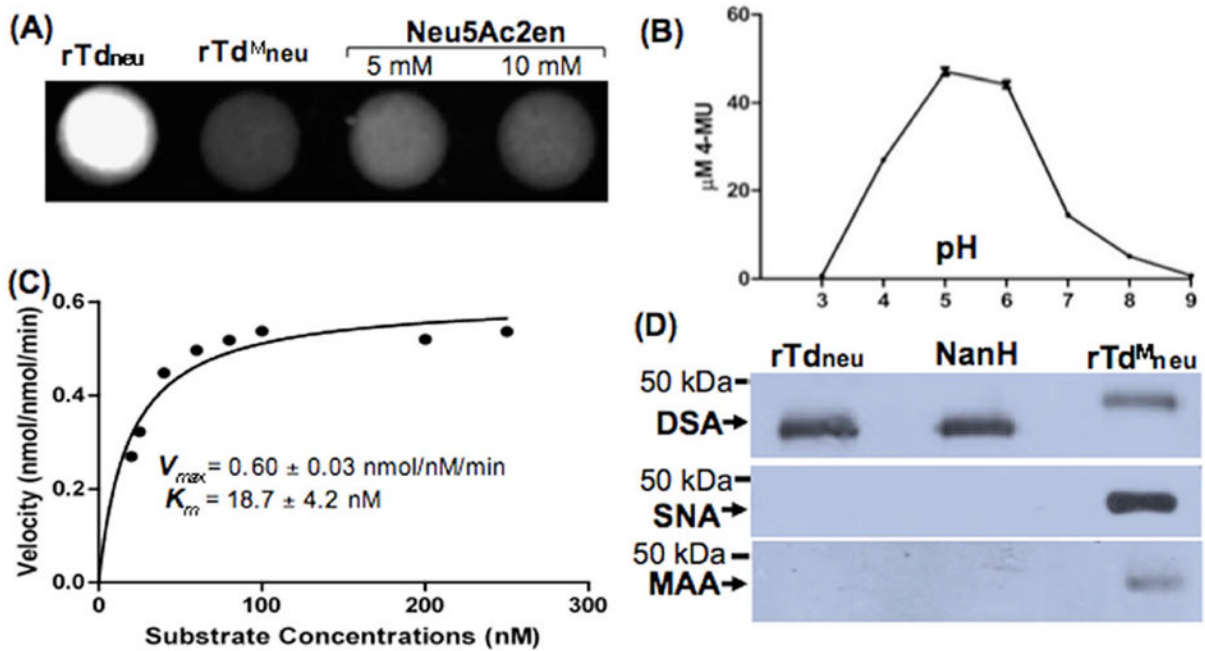


Figure 1. TDE0471 (Td_{neu}) enzymatic assay

(A) Filter paper spot sialidase test of rTd_{neu}. The substrate 4-MUNANA was treated with: rTd_{neu} (spot 1, from the left), truncated rTd_{neu} (rTd^M_{neu}, spot 2), and Neu5Ac2en (spots 3 and 4). The image was processed using the ChemiDoc XRS system (Bio-Rad) with an excitation wavelength of 302 nm and an emission wavelength of 548 nm. (B) rTd_{neu} is active in a wide pH range. For this assay, the spot test was carried out from pH 3.0 to pH 9.0. The release of 4-MU (the product 4-MUNANA) was quantified based on a standard curve (fluorescence intensity vs. defined amounts of 4-MU). The data shown are the means from at least three samples. Error bars represent \pm the standard error of the mean (SEM). The amounts of 4-MU (the product of 4-MUNANA) were determined using a standard curve. (C) Kinetic analysis of rTd_{neu}. This assay was carried out according to a standard method for enzyme kinetics studies. The saturation curves were fitted to Michaelis-Menten kinetics using GraphPad Prism (GraphPad Software), and K_m and V_{max} were calculated. (D) Lectin blot analysis of rTd_{neu}. The assay was performed as previously described (Gut *et al.*, 2008; Li *et al.*, 2012). Biotin-labeled SNA, MAA, and DSA lectins were used to detect terminal 2,6- and 2,3-linked sialic acid and galactose linked to GlcNAc of human -1 acid glycoprotein (AGP), respectively. The *C. perfringens* neuraminidase NanH was used as a positive control, and rTd^M_{neu} was used as a negative control. Arrows point to the products detected by the three lectins.

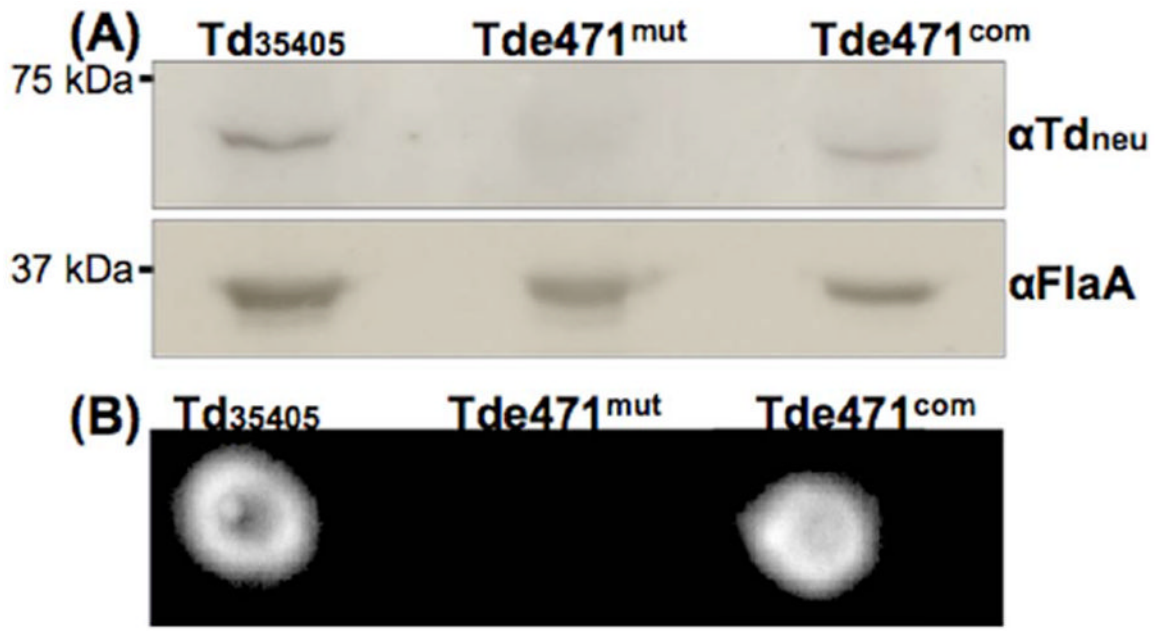


Figure 2. Characterizations of the *TDE0471* mutant (*Tde471^{mut}*) and its complemented strain (*Tde471^{com}*)

(A) Immunoblotting analysis of Td₃₅₄₀₅, Tde471^{mut}, and Tde471^{com} strains. Equivalent amounts of Td₃₅₄₀₅, Tde471^{mut}, and Tde471^{com} whole cell lysates were analyzed by SDS-PAGE and then probed with αTd_{neu} , a specific antibody against Td_{neu}. The flagellin protein, FlaA, was used as a sample loading control and detected by $\alpha FlaA$, a specific antibody against FlaA. (B) Filter paper spot test of Td₃₅₄₀₅, Tde471^{mut}, and Tde471^{com} strains. The assay was carried out as described in Figure 1A using the whole cell lysates of the three strains.

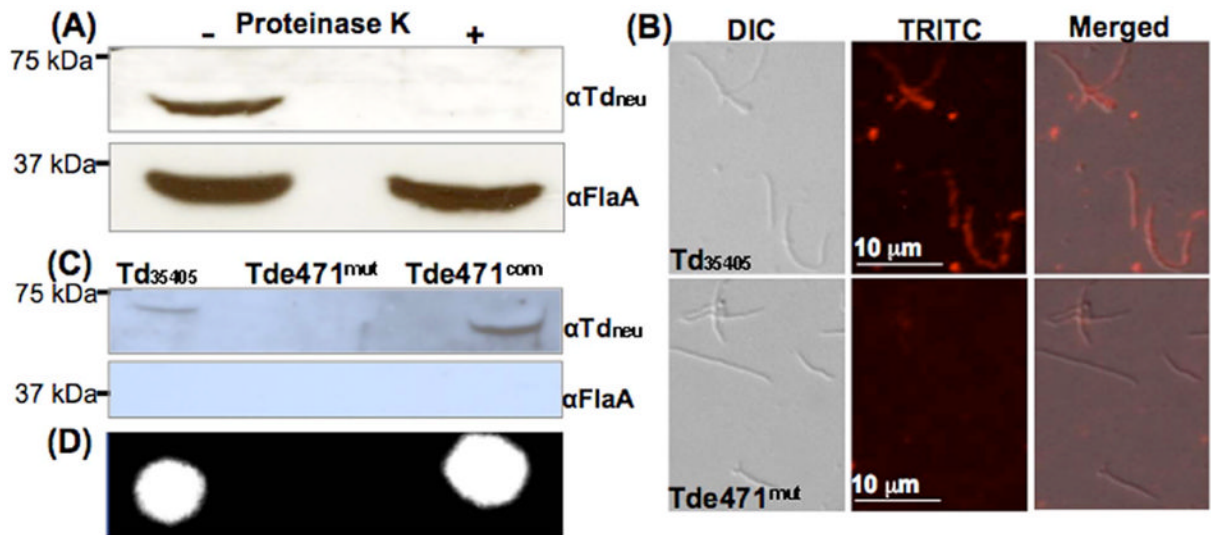


Figure 3. Localization of Td_{neu}

(A) Immunoblotting analysis of proteinase K-treated Td₃₅₄₀₅ whole-cell lysates. The spirochetes were either incubated with proteinase K (240 μg/ml) or PBS at 37°C for 1 h. The resultant samples were separated on SDS-PAGE and then probed with Td_{neu} and FlaA.

(B) IFA analysis. Td₃₅₄₀₅ and Tde471^{mut} cells were fixed with methanol, stained with Td_{neu}, and counterstained with a goat-anti-rat Texas red antibody as previously described. The micrographs were taken under DIC light microscopy or fluorescence microscopy with a tetramethylrhodamine isothiocyanate (TRITC) emission filter, and the resultant images were merged. (C) Detection of Td_{neu} in the supernatants prepared from the cultures of Td₃₅₄₀₅, Tde471^{mut}, and Tde471^{com} strains by immunoblotting analysis. FlaA was used as a sample preparation control. (D) Detecting neuraminidase activity in the supernatants by the filter paper spot test as described in Figure 1A.

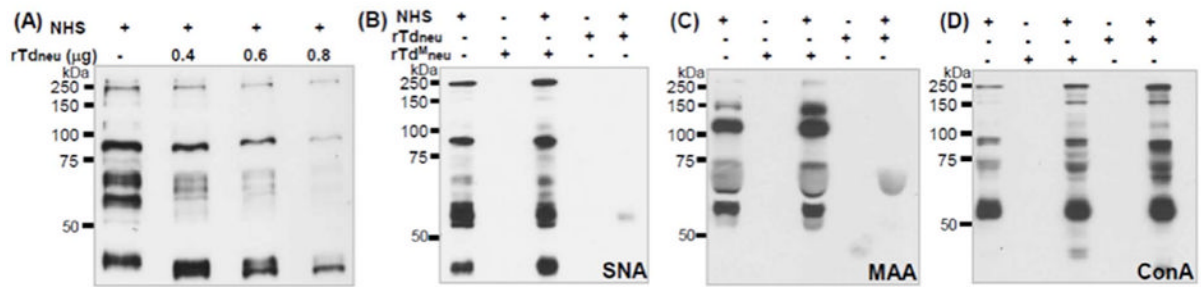


Figure 4. Lectin blot analysis of rTd_{neu}-treated human serum

(A) Normal human serum (NHS) was treated with various amounts of rTd_{neu} at 37°C for 1 h. The resultant samples were separated on SDS-PAGE gels followed by Coomassie blue staining; (B-D) The NHS was treated with 0.2 µg rTd_{neu} or the same amount of rTd_{neu}^M at 37°C for 3 hours. The resultant samples were separated on SDS-PAGE gels, transferred to PVDF membranes, and probed with biotin-labeled SNA (B, 0.2 µg/ml), MAA (C, 2 µg/ml), or ConA (D, 0.5 µg/ml). The final concentrations of NHS in the reactions were 0.15% for SNA and ConA, and 1.5% for MAA.

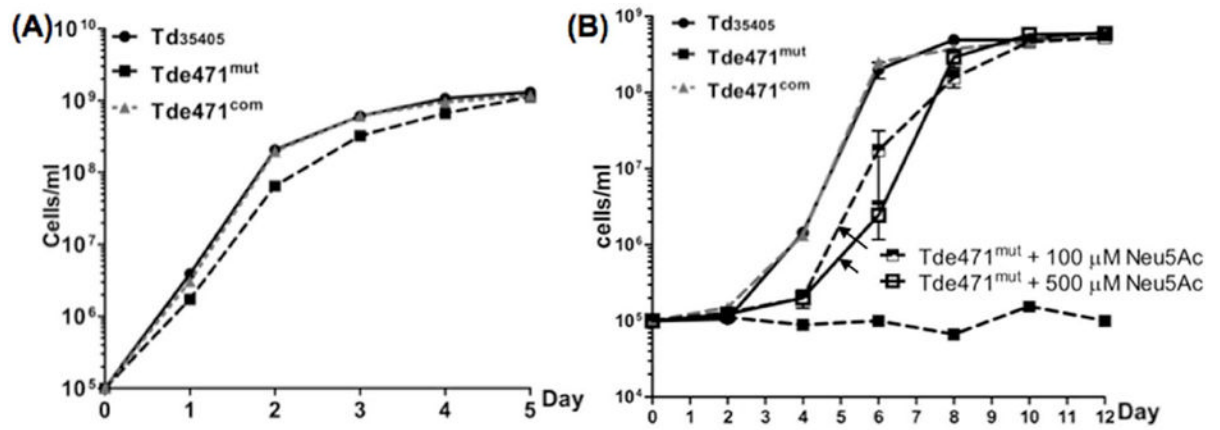


Figure 5. Growth curves of Td₃₅₄₀₅, Tde471^{mut}, and Tde471^{com} strains in TYGVS medium (A) and the serum growth medium (B)

The serum growth medium contains 50% heat-inactivated rabbit serum supplemented with 6.0 μg/ml TPP. Cell counting was repeated in triplicate with at least three independent samples. The results are expressed as the mean ± SEM. The data were analyzed by one-way ANOVA followed by Tukey's multiple comparison at $p < 0.01$.

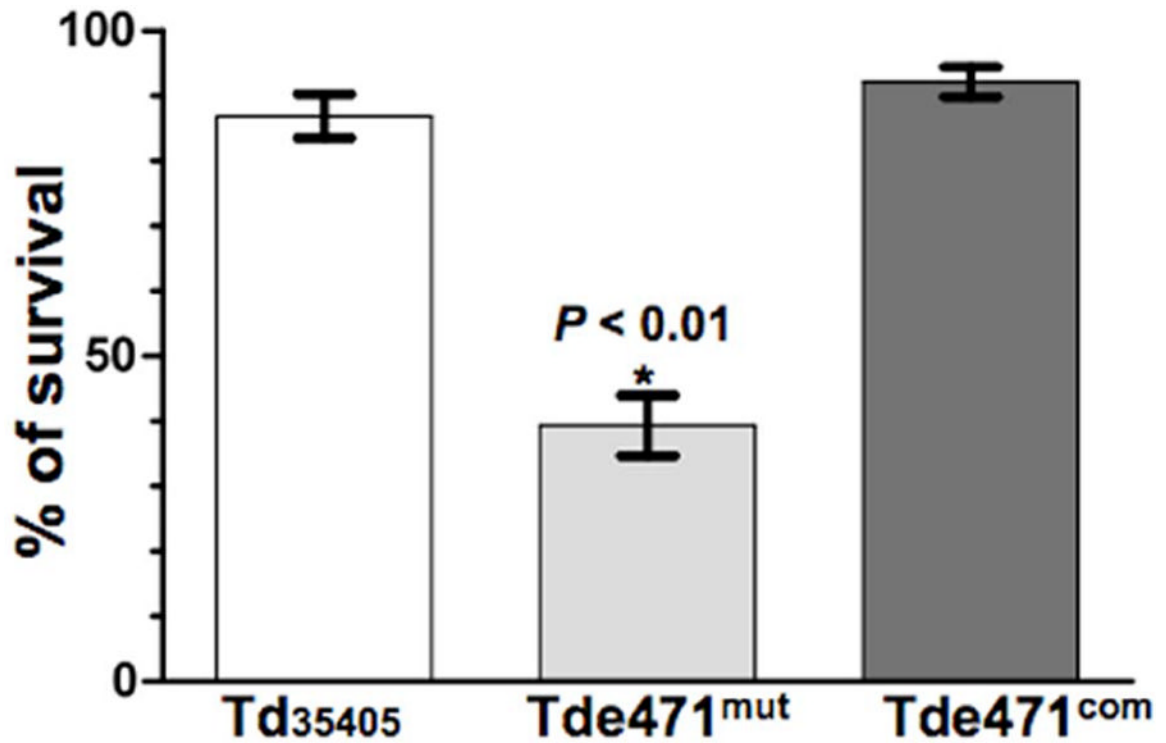


Figure 6. Survival rates of Td₃₅₄₀₅, Tde471^{mut}, and Tde471^{com} strains in serum

The strains were co-incubated with either 25% normal human serum (NHS) or heat-inactivated human serum (HIS) for 1 h at 37°C. The number of surviving bacteria was counted using a Petroff Hausser counting chamber. The survival rates were calculated as follows: the total number of living cells in NHS divided by the total number of living cells in HIS. Cell counting was repeated in triplicate with at least three independent samples, and the results are expressed as the mean \pm SEM. The data were analyzed by one-way ANOVA followed by Tukey's multiple comparison at $p < 0.01$.

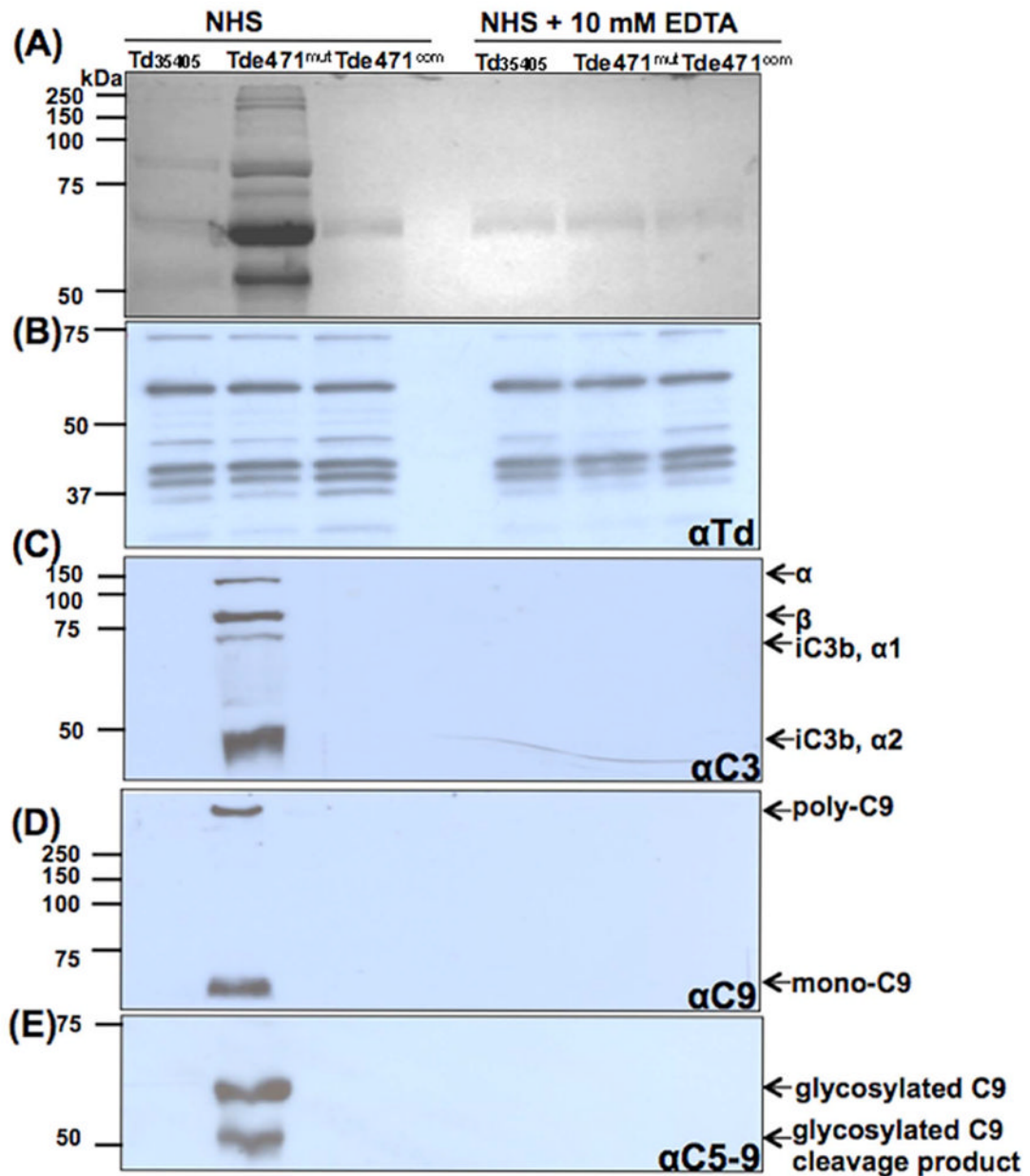


Figure 7. Deposition of membrane attack complex (MAC) on Td₃₅₄₀₅, Tde471^{mut}, and Tde471^{com} strains

10⁶ cells of Td₃₅₄₀₅, Tde471^{mut}, or Tde471^{com} were co-incubated with 25% NHS for 20 min at 37°C. As a control, 10 mM EDTA was added to the reactions to block complement activation. The resultant serum-treated cells (approximately 5 × 10⁴) were subjected to SDS-PAGE, followed by coomassie blue staining (A), or probed with four different antibodies as labeled: (B) Td, a rat polyclonal antibody against Td₃₅₄₀₅; (C) C3, a polyclonal antibody against C3; (D) C9, a monoclonal antibody against C9; and (E) C5-9, a monoclonal antibody against C5-9 complex. Notably, complement components were detected only in the serum-treated Tde471^{mut}, but not in the serum-treated Td₃₅₄₀₅ and Tde471^{com} strains. The complement factors detected by the antibodies and their molecular weights are indicated.

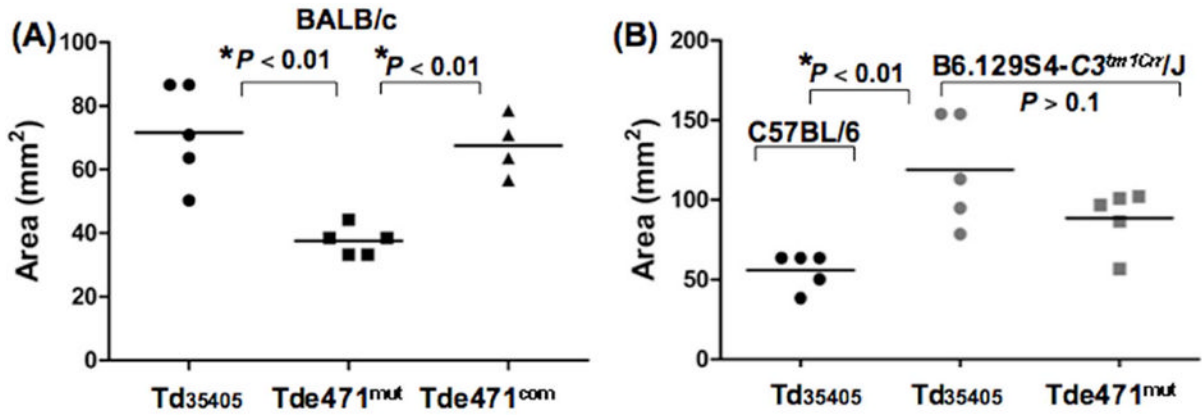


Figure 8. Evaluating the virulence of Td₃₅₄₀₅, Tde471^{mut}, and Tde471^{com} strains using a mouse-skin-infection model

The infectious studies were carried out as previously documented (Kesavalu *et al.*, 1997). 10^9 bacterial cells were subcutaneously injected into each mouse. Three different mouse strains were included: (A) BALB/C mice; (B) The complement-deficient mice (B6.129S4-C3^{tm1Crj}/J) and its parental wild-type C57BL/6 mice. Ten days post-infection, the sizes of the abscesses were measured. The average sizes of the observed abscesses were calculated. The statistic differences between groups were analyzed by t-test, two-tailed at $p < 0.01$